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We have discovered a mechanism of c-Src synergy with the EGFR and located specific points at which the pathway can be interdicted. Specifically, we have shown that kinase-inactive c-Src is able to inhibit tumorigenicity of the 10T1/2 mouse fibroblast model cells by not phosphorylating the receptor on Tyr 845 in the activation loop of the kinase. The phosphorylation of Tyr 845 is required for EGF and serum-induced DNA synthesis through the EGFR. This phosphorylation site in cancer cells presents an appealing target for cancer therapy of tumors that overexpress the EGFR and c-Src, such as breast cancer. We have also shown that inhibition of c-Src in breast tumor cells efficiently blocks tumor formation further supporting that this may be an effective therapeutic target. Finally, in an effort to isolate a single domain of c-Src to target, we have demonstrated that the SH2 domain and surprisingly the kinase domain of c-Src have inhibitory effects on tumorigenicity and growth of breast tumor cells.

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David A Tice 7/28/98

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INTRODUCTION

Considerable evidence has accumulated in recent years to suggest that c-Src and members of the EGF receptor family are critical elements in the etiology of multiple human cancers. Both kinases are found overexpressed in many of the same types of tumors, including glioblastomas and carcinomas of the colon, breast, and lung (1-4), raising the question of whether they functionally interact to promote the growth of these malignancies. In breast cancer, overexpression of EGFR family members is estimated to occur in 60% or more of the cases (5), and overexpression of the family member, HER2/*neu*, has been associated with a poor prognosis for the disease (6). Recent reports have also described overexpression of c-Src in a significant majority of patients with breast cancer, a frequency that approaches 100% (1). Studies to assess the oncogenic potential of each kinase have shown that the EGFR is tumorigenic when overexpressed in cultured fibroblasts and activated by ligand (7, 8), but overexpression of c-Src alone is insufficient for malignant transformation (9, 10).

A possible role for c-Src in tumorigenesis was revealed when it was demonstrated in C3H10T1/2 murine fibroblasts that co-overexpression of c-Src and the EGFR resulted in a synergistic increase in EGF-induced DNA synthesis, growth in soft agar, and tumorigenesis, as compared to cells overexpressing either the EGFR or c-Src alone (11). This co-operation correlated with the EGF-dependent formation of a physical complex

between c-Src and the EGFR (11), the appearance of two novel sites of tyrosine phosphorylation (Y845 and Y1101) on the c-Src-associated EGFR, and increased phosphorylation of receptor substrates (11). These results suggest that one mechanism by which c-Src could augment the mitogenic/tumorigenic activity of the receptor is by associating with and hyper-activating the receptor via phosphorylation of novel tyrosine residues. Co-overexpression, co-association, and phosphorylation of Y845 and Y1101 have also been observed in human tumor cells (12-14; Biscardi *et al.*, submitted), suggesting that synergism between c-Src and the EGFR may occur in a subset of human tumors as well as in murine fibroblasts.

To determine if phosphorylation of Y845 or Y1101 is critical to the biological synergy between c-Src and the EGFR and to identify which of the two kinases is responsible for mediating the phosphorylations, we analyzed a panel of murine fibroblasts that overexpressed either *wt* c-Src (K+ c-Src) or kinase defective c-Src (K- c-Src) alone or together with the EGFR for growth properties and the presence of a stable complex containing the EGFR and c-Src. We found that K- c-Src inhibits EGF-dependent growth in soft agar and tumorigenesis in nude mice even though it is still capable of associating with the receptor. However, K- c-Src was unable to mediate the phosphorylation of Y845 on the receptor. As a direct test of the requirement of this phosphorylation for receptor function, we engineered a variant receptor harboring a Y845F mutation in the EGFR and observed that this mutated receptor ablated EGF and serum-induced DNA synthesis. The data support a model wherein phosphorylation of Y845 on the EGFR by c-Src is required for EGF-induced mitogenesis and tumorigenesis.

BODY

Materials and Methods

Cell Lines. The derivation, characterization, and maintenance of the clonal C3H10T1/2 murine fibroblast cell lines Neo (control), K+ (*wt* chicken c-Src overexpressors), K- (A430V kinase deficient, chicken c-Src overexpressors), EGFR (*wt* human EGFR overexpressors), and EGFR/K+ (*wt* EGFR/*wt* c-Src double overexpressors) have been described previously (10, 11, 15). EGFR/K- (*wt* EGFR overexpressors/kinase deficient c-Src) cell lines were derived by infection of K- cells with a recombinant amphotropic retrovirus encoding the human EGFR (8), cloning by limiting dilution, and screening for overexpression of the receptor and maintenance of K- c-Src by Western immunoblotting. Clonal cell lines used in this study were estimated to express 25,000 - 60,000 human EGF receptors/cell, based on comparative Western blotting analysis that used as a standard a 10T1/2 cell line that by Scatchard analysis was determined to express approximately 200,000 receptors/cell (5HR11 cells) (11). Clones included $EGFR_5$, $EGFR_8$, $EGFR_{27}$, $EGFR/K+_8$, $EGFR/K+_9$, $EGFR/K+_{10}$, and $EGFR/K-_2$, $EGFR/K-_5$, $EGFR/K-_{41}$, and $EGFR/K-_{56}$. K+ and K- c-Src overexpression was estimated to be 20 - 25 fold over endogenous.

The maintenance of the breast tumor cell lines have been described previously (14). Stable cell lines expressing K- c-Src or any of the c-Src domains were generated by transfection with Lipofectin (GibcoBRL, Gathersburg MD) followed by limiting dilution in media containing neomycin.

Constructs. A pcDNA3 vector (Invitrogen, Carlsbad, CA) encoding human EGFR with a Y845F mutation was constructed by inserting a DraIII-BstEII fragment containing the Y845F mutation (from plasmid pCO11, gift of L. Beguinot) into the corresponding DraIII-BstEII site of pcDNA3 encoding *wt* EGFR (gift of S. Decker). C-Src domains were isolated by PCR and cloned into pcDNA3.1 myc tag vector (Invitrogen, Carlsbad, CA).

Western Immunoblotting. Western blot analysis was performed as previously described (11, 15), using Ab-4 rabbit polyclonal antibody (Calbiochem, La Jolla CA) or F4 mouse monoclonal antibody (Sigma, St. Louis MO) to detect the EGFR, purified 2-17 mouse monoclonal antibody (mAb) (Quality Biological, Inc., Gaithersburg MD) or EC10 mouse mAb ascites (prepared in our laboratory and used at a 1:10,000 dilution) to identify c-Src, polyclonal anti-SHC antibody (Upstate Biotechnology, Lake Placid NY) to visualize SHC, B3B9 anti-MAPK mouse monoclonal antibody (16) to detect MAPK, and anti-myc epitope tag mouse monoclonal antibody (Upstate Biotechnology, Lake Placid NY) to identify the myc-tagged c-Src domains. [^{125}I]-protein A (ICN, Costa Mesa CA) or [^{125}I]-labeled goat anti-mouse immunoglobulin (New England Nuclear, Boston, MA) and autoradiography were employed to localize binding of primary antibodies.

Colony Formation in Soft Agar and Tumorigenicity. Anchorage-independent growth was measured as previously described (16). 1 x 10^5 cells were plated for the 10T1/2 clones whereas 2 x 10^4 cells were plated for the breast tumor cell lines. PP1 was used at a final concentration of 10μM and supplemented every 2-4 days. Colonies were stained for 20 hr at 37 °C in a solution of iodonitrotetrazolium salt (1μg/ml; Sigma, St. Louis MO) in water and counted using EagleSight analysis software (Stratagene, La Jolla CA). The soft

agar colony data for the 10T1/2 clones include analysis of three separate clones for each cell type, $EGFR_5$, $EGFR_8$, $EGFR_{27}$, $EGFR/K+_8$, $EGFR/K+_9$, $EGFR/K+_{10}$, $EGFR/K-_2$, $EGFR/K-_5$, and $EGFR/K-_{56}$. Assessment of tumor formation in Taconic nu/nu mice was performed as previously described (11).

***In vitro* Kinase Assay, Metabolic Labeling with $^{32}P_i$, and Two-Dimensional Tryptic Phosphopeptide Analysis.** Methods for immunoprecipitation, *in vitro* kinase assay, metabolic $^{32}P_i$-labeling, and two-dimensional phosphopeptide analysis have been described (11). In the metabolic labeling experiments, 5 uM pervanadate and 3 mM H_2O_2 were added to cells simultaneously with 100 ng/ml EGF and incubated for 5 min before harvesting.

Transient Transfections and BrdU Incorporation. K+ cells or Neo control cells were transiently transfected with 30μg Superfect (Qiagen, Chatsworth CA) and 4μg vector, *wt* EGFR, or Y845F EGFR plasmid DNA according to manufacturer's directions and incubated in a humidified, 37^0C, 5% CO_2 atmosphere for 48 hr to allow a confluent monolayer to form. Transfected cells were then serum-starved for 30 hr prior to addition of 100μM BrdU and either 40 ng/ml EGF or 10% FBS in growth medium, at which time they were incubated for an additional 18 hr and co-stained for human EGFR expression and BrdU incorporation as described by the manufacturer of the BrdU-specific mAb (Boehringer-Mannheim, Indianapolis IN). Specifically, fixed cells were treated with 2N HCl for 1 hr at 37°C and incubated with a mixture of primary antibodies (1:100 dilution of EGFR-specific Ab-4 and a 1:15 dilution of anti-BrdU mouse mAb), followed by incubation with a mixture of secondary antibodies (75 μg/ml FITC-conjugated goat anti-

rabbit IgG and 4μg/ml texas red-conjugated goat anti-mouse IgG, both from Jackson Immunoresearch Laboratories, West Grove, PA).

Similarly, MDA-MB468 cells were transfected using 20μl of Lipofectin and 4 μg of plasmid DNA containing myc tagged c-Src domains per 35 mm well. Cells were allowed to recover for 24 hr and then were incubated with 100μM BrdU in the presence of 10% FBS for 17 hr. Co-staining for expression of the myc-epitope tag using 1:500 rabbit polyclonal anti-myc tag antibody (Upstate Biotechnology, Lake Placid NY) and BrdU incorporation is detailed above.

Results and Discussion

To determine if phosphorylation of Y845 or Y1101 were mediated by c-Src or the EGFR, clonal 10T1/2 fibroblasts that stably overexpress *wt* EGFR and K- chicken c-Src were created from a stable K- c-Src expressing line, as described in Materials and Methods. Figure 1, panel a shows the levels of receptor and c-Src that are expressed in the various cell lines used in this study. EGFR/K- clones (lanes 6-8) expressed levels of receptor comparable to those in EGFR/K+ (lane 4) and EGFR (lane 2) lines, while all clones expressing K- c-Src (lanes 5-8) contained comparable levels of c-Src as those clones expressing K+ c-Src (lanes 3, 4). Figure 1, panel b shows that the EGFR/K- clones exhibited diminished anchorage independent growth in the presence of EGF compared to EGFR/K+ double overexpressors, demonstrating a requirement for the kinase activity of c-Src for potentiation of EGF-induced soft agar growth. Moreover, relative to cells overexpressing EGFR alone, the EGFR/K- clones also showed reduced soft agar growth, indicating that K- c-Src can function in a dominant negative fashion for EGFR-induced colony formation. The dominant negative effect was manifested by both

reduced number (Fig. 1, panel b) and significantly smaller average size (Fig. 1, panel c) of the EGFR/K- colonies as compared to those of EGFR/K+ or EGFR cells. As previously reported, Neo control and K+ c-Src cells produced no or significantly fewer colonies than EGFR cells (11). K- c-Src cells also gave no colonies (data not shown). Table 1 shows that the growth of tumors *in vivo* was completely ablated in mice injected with EGFR/K- cells compared with EGFR or EGFR/K+ cells, demonstrating that K- c-Src has an even stronger dominant negative effect on tumor growth *in vivo* than on growth in soft agar. Together these results underline the requirement for c-Src kinase activity in both the potentiating effect of overexpressed *wt* c-Src and the ability of overexpressed EGFR alone to induce oncogenic growth.

To determine if K- c-Src might be eliciting its biological effects through the receptor, we examined the association between the two kinases, using an immune complex *in vitro* kinase assay as previously described (11). C-Src was immunoprecipitated from the 10T1/2 clones using a chicken c-Src-specific antibody, EC10, to minimize recognition of endogenous c-Src and to determine if the exogenously expressed K- c-Src could interact with the EGFR. An EGF-sensitive *in vitro* phosphorylation of a ~170 kD protein was observed in the c-Src immunoprecipitates prepared from EGFR/K- (Fig. 2, panel a, lanes 20, 23, and 26) as well as from EGFR/K+ cells (lanes 11 and 14). These results demonstrate that c-Src kinase activity is not required for association and suggest that K- c-Src may be eliciting its dominant negative effects (at least in part) directly through the receptor, since the association is still intact.

As described before (11), two phosphotryptic peptides appear in the map of *in vitro* phosphorylated receptor associated with K+ c-Src (Fig. 3, panel b) that are either

absent or present in reduced amounts in the map of "free", activated receptor (panel a). These peptides contain Y845 and Y1101, whose identification is described in Biscardi *et. al.* (submitted). In contrast to the receptor associated with K+ c-Src, phosphorylation of Y845 was virtually absent in receptor associated with K- c-Src (Fig. 3, panels c and d), while the level of Y1101 phosphorylation was somewhat reduced. Similar results were observed in $^{32}P_i$ metabolic labeling experiments (panels e and f). Phosphorylation of Y845 and Y1101 were either undetectable or decreased in receptor immunoprecipitated from EGFR/K- cells (panel f) as compared to that from EGFR/K+ (panel e) cells. These results indicate that phosphorylation of Y845, and to a lesser extent of Y1101, is dependent on the kinase activity of c-Src, both *in vitro* and *in vivo*.

The position corresponding to Y845 is highly conserved among serine/threonine and tyrosine kinases and is situated in the activation loop between subdomains VII and VIII (17). Three-dimensional structural studies of several kinases have pointed to the importance of phosphorylation of this residue in stabilizing the activation loop in a conformation favorable for substrate and ATP binding (18-20). In agreement with the structural data, mutational analysis of the corresponding residue in tyrosine kinase receptors has shown a requirement for phosphorylation of this residue for full biological function in response to ligand (21-25). Y845 homologues in other tyrosine kinase receptors have all been shown to be autophosphorylation sites. In contrast, Y845 of the EGFR has not been identified as such, and its importance to EGFR function has not been ascertained. The failure to identify Y845 as a site of autophosphorylation may reflect either the highly labile nature of the phosphorylation or the c-Src-dependency of the phosphorylation (26; Biscardi *et al.*, submitted).

To test whether phosphorylation on Y845 is important for the mitogenic function of the EGFR, we transiently transfected a Y845F mutant or *wt* receptor into K+ cells or Neo control cells and assessed mitogenesis by measuring EGF-induced BrdU incorporation into newly synthesized DNA. In contrast to the *wt* receptor, the Y845F mutant was unable to stimulate DNA synthesis upon EGF treatment (Fig. 4). Indeed, the reduced level of BrdU incorporation, which approached that of serum-starved cells, indicated that Y845F EGFR is capable of interfering with signaling through endogenous receptors, thereby acting in a dominant negative fashion. Similar results were obtained in Neo control cells (data not shown). These data support the hypothesis that phosphorylation of Y845 is required for the EGF-induced mitogenic function of the receptor.

Surprisingly, the Y845F variant of the EGFR also inhibited serum-induced DNA synthesis in a dominant negative manner (Fig. 4). The mechanism of this inhibition is unclear at the present time. However, the EGFR has recently been shown to play an essential role in signaling through G protein coupled receptors (GPCR) (27), and the Src family of tyrosine kinases has also been directly implicated in GPCR-mediated MAP kinase activation (28, 29). C-Src is thought to be responsible for phosphorylating the EGFR in response to GPCR activation (30), leading to the generation of docking sites for Shc and Grb2 and activation of the Ras/MAPK pathway. The major mitogenic component of serum is lysophosphatidic acid (LPA), a ligand for GPCR (31). Therefore, one possible mechanism by which the Y845F EGFR could prevent serum-induced BrdU incorporation might be the inability to phosphorylate Y845 via a GPCR route.

To further characterize the Y845F mutation in the EGFR, we measured the mutants ability to autophosphorylate and to phosphorylate and activate members of the SHC-RAS-MAPK pathway. In transient transfection assays in COS-7 cells, immunoprecipitated mutant receptor had as much autophosphorylation activity as the wild type kinase (Fig. 5). Due to the high levels of overexpression in COS-7 cells, both forms of the receptor are activated in the absence of EGF due to constitutive dimerization. The mutant EGFR also retained the ability to phosphorylate co-transfected HA-SHC and flag-MAPK to equal levels as wild type (Figs. 6 & 7). These results suggest that this mutation in the EGFR differs from the other growth factor receptors in its ability to autophosphorylate and suggests that the inhibition of DNA synthesis occurs independent of the RAS-MAPK pathway. We are currently searching for differences in signaling via the STAT family of transcription factors.

The data presented here provide a mechanism for c-Src's role in EGF and possibly GPCR-mediated DNA synthesis and tumorigenesis. Phosphorylation of Y845 on the EGFR by a c-Src-mediated event is required for EGF-induced DNA synthesis. Based on the findings that Y845 is not phosphorylated by the *wt* receptor alone (Fig. 3) and that the kinase activity of c-Src is required for phosphorylation of Y845, we conclude that c-Src is the most likely kinase to phosphorylate the receptor. Interruption of this phosphorylation by overexpressing a kinase-deficient c-Src or a Y845F mutant of the EGFR blocks signaling and thus growth.

These findings have direct implications for the etiology of human cancers. In tumor cells that overexpress both c-Src and the EGF receptor, we postulate that the probability of Y845 phosphorylation increases, an event that results in promotion of

growth and anchorage independence. Since phosphorylation of Y845 has been shown to occur in cultured human tumor cells that overexpress c-Src (Biscardi *et al.*, submitted), the above paradigm may have relevance for the disease *in situ*. Development of methods to inhibit the ability of c-Src to phosphorylate Y845 may result in a novel, more "tumor-specific" treatment for cancers such as carcinomas of the breast, colon, and lung.

In order to assess the requirement for c-Src in growth of such carcinomas, we treated several different breast tumor cell lines with a Src family inhibitor, PP1, and measured their ability to grow in soft agar. Treatment with PP1 dramatically inhibited colony formation of three different breast tumor cell lines (Fig. 8), suggesting a universal requirement for c-Src in the ability of these cells to grow in anchorage independent conditions.

As a separate measure of c-Src's requirement for tumorigenicity in these cells, K-c-Src was stably transfected into two different breast tumor cell lines, MDA-MB468 and MCF-7 cells. Two clones in each cell line that expressed different amounts of K- c-Src were chosen for further analysis (Fig. 9a). The expression of K- c-Src was able to dose-dependently inhibit soft agar colony formation of both breast tumor cell types (Fig. 9b). This inhibition was replicated *in vivo* in tumor formation in nude mice (Table II), further supporting the requirement for c-Src in the maintenance of the tumorigenic phenotype of these cells. This also supports the hypothesis that c-Src may be an effective therapeutic target in breast cancer.

To determine which domain of c-Src is responsible for the inhibition of tumorigenicity in the breast tumor cells and thus be a more specific therapeutic target, we generated several myc-tagged constructs (Fig. 10). Stable clones of MDA-MB468 cells

expressing each one of these constructs were generated and screened for levels of overexpression (Fig. 11). All of the domains were overexpressed at levels 3-8 fold above endogenous c-Src except the kinase domain which could only be observed at levels comparable to endogenous (data not shown). The inability of the c-Src kinase domain to be overexpressed in these cells was irrespective of the activity since both the kinase active and the kinase-inactive domains could not be expressed above endogenous c-Src levels (data not shown).

To determine if any of the c-Src domains were able to inhibit achorage independent growth, two MDA-MB468 clones of each domain were tested for their ability to form colonies in soft agar. The SH2 domain and interestingly one of the two clones of K- domain inhibited soft agar growth about 50% while the other domains had little to no effect (Fig. 12). Several other clones expressing K- domain were assayed for growth in soft agar with 50% of the clones tested showing inhibition (data not shown). The clonal variablity of the K- clones may be due to compensating mutations that may overcome the low expression of the construct. However, in the K- clones that show inhibition, the magnitude of this inhibition is heightened when considering the low levels of K- domain overexpression.

The mechanism of inhibition for the SH2 domain is likely mediated through P-Tyr binding of target proteins, thereby saturating out critical signal transducers. The mechanism of inhibition of the kinase domain is more difficult to predict since a non-catalytic role for a kinase domain has not been identified. However, there is a report that c-Src binds the EGFR through a region in its kinase domain (32). The c-Src kinase domain could be inhibiting soft agar colony formation by interfering with signaling

through growth factor receptors. However, no changes in MAPK activity upon EGF stimulation could be observed in cells expressing K- c-Src (data not shown). In addition no changes in phosphorylation of other known or predicted c-Src substrates could be observed including p190RhoGAP, p130CAS, cortactin, paxillin, SHPTP1, SHPTP2, α or β catenin (data not shown). We are currently exploring other options at this time.

Since we were unable to detect overexpression of the kinase domain in stable clones, we wanted to determine if expression of the domain was detrimental to the cell. MDA-MB468 cells were transiently transfected with the various c-Src domains, allowed to recover for two days, and then assayed for their ability to undergo DNA synthesis by measuring BrdU incorporation under normal growth conditions. The same pattern exists in the BrdU incorporation assay as was seen in the soft agar assay, that is, the SH2 and kinase domains were most effective at inhibiting DNA synthesis (Fig. 13). The lack of stable overexpression of the kinase domain may be due to the strong inhibition of DNA synthesis of cells grown in the presence of serum. The inability to undergo DNA synthesis was not due to the induction of apoptosis as measured by a TUNEL assay 48 hours post-transfection with any of the c-Src domains (data not shown).

Relevance to the Statement of Work

The work discussed above represents the near completion of Specific Aims I and II as originally designed.

CONCLUSIONS

We have detailed a mechanism of c-Src synergy with the EGFR and located specific points at which the pathway can be interdicted. Specifically, we have shown that kinase-inactive c-Src is able to inhibit tumorigenicity of the 10T1/2 mouse fibroblast model cells by not phosphorylating the receptor on Tyr 845 in the activation loop of the kinase. This phosphorylation of Tyr 845 is required for EGF and serum-induced DNA synthesis through the EGFR. The phosphorylation of this site in cancer cells presents an appealing target for cancer therapy of tumors that overexpress the EGFR and c-Src, such as breast cancer. We have also shown that inhibition of c-Src in breast tumor cells efficiently blocks tumor formation further supporting that this may be an effective therapeutic target. Finally, in an effort to isolate a single domain of c-Src to target, we have demonstrated that the SH2 domain and surprisingly the kinase domain of c-Src have inhibitory effects on tumorigenicity and growth of breast tumor cells.

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APPENDIX

Figure Legends

1.) **Figure 1. Dominant repressive effect of K- c-Src on EGF-induced soft agar colony formation. a,** Western immunoblot analysis of C3H10T1/2 murine fibroblast clonal cell lines stably overexpressing EGFR and either *wt* (K+) or kinase deficient (K-) c-Src. **b,** Values for number of colonies are the mean ± SEM of at least 6 experiments in which 10^5 cells of each clone were seeded per plate in triplicate. *P<0.04 and **P<0.002 compared to EGFR. **c,** Photomicrographs of representative fields of soft agar colonies formed from the indicated cell lines were taken after two weeks growth.

Figure 2. K- c-Src associates with the EGFR. Confluent, serum-starved cells were untreated or treated with EGF (100 ng/ml) for 15 min, and extracts were immunoprecipitated with either chicken c-Src-specific mAb EC10 (designated +) or a negative control mouse IgG (-). **a,** Immunecomplexes were subjected to an *in vitro* kinase assay using γ-[^{32}P]-ATP, and phosphorylated products were analyzed by SDS/PAGE and autoradiography. The region of the autoradiograph around the 170 kDa products is shown. **b,** The amount of c-Src in each precipitate was visualized by Western immunoblot analysis with EC10 mAb. K- c-Src in EGFR/K- clones was verified to be catalytically inactive in the immune complex kinase assay (data not shown).

Figure 3. Y845 is not phosphorylated in EGFR complexed with K- c-Src. Panels a-d: The 170 kD bands that were phosphorylated *in vitro* (as in Fig. 2) in c-Src (panels **b-d**) or receptor (panel **a**) immunecomplexes prepared from the indicated cell lines were excised, digested with trypsin, resolved by 2-D electrophoresis/chromatography, and subjected to autoradiography. The positions of peptides containing Y845 and Y1101, which were identified previously (Biscardi *et al.,* submitted), are indicated. **Panels e and f**: Receptor immunoprecipitates from the indicated cell lines that had been metabolically labeled with $^{32}P_i$ were analyzed as in panels a-d. Equal cpms were loaded in panels a-d and in panels e vs. f.

Figure 4. Phosphorylation of Y845 is essential for EGFR function. K+ cells were transfected with plasmid DNA encoding Y845F or *wt* EGFR, cultured for two days, serum starved for 30 hr, and left untreated or treated with either 40 ng/ml EGF or 10% serum for 18 hr. 100 μM BrdU was added to all cells at the time of mitogen addition. Cells were fixed and co-stained for EGFR expression and BrdU incorporation. Results

are expressed as the mean percent ± SEM of cells expressing EGFR that were positive for BrdU incorporation. Thirty-five to seventy-five cells were analyzed for each variable in 3 independent experiments.

Figure 5. Y845F mutant receptor retains its ability to autophosphorylate. Cos-7 cells were transfected with plasmid DNA encoding Y845F or *wt* EGFR, cultured for two days, serum starved overnight, and left untreated or treated with 100ng/ml EGF for 5 min. Extracts were immunoprecipitated with m108 monoclonal antibody. **a.** Immunecomplexes were subjected to an *in vitro* kinase assay using γ-[^{32}P]-ATP, and phosphorylated products were analyzed by SDS/PAGE and autoradiography. **b.** The amount of EGFR in each precipitate was visualized by Western immunoblot analysis with clone F4 monoclonal antibody.

Figure 6. Y845F mutant receptor retains its ability to phosphorylate SHC. Cos-7 cells were transfected with plasmid DNA encoding HA-SHC and either Y845F or *wt* EGFR, cultured for two days, serum starved overnight, and left untreated or treated with 100 ng/ml EGF for 10 min. Extracts were immunoprecipitated with 12CA5 anti-HA antibody and resolved by SDS/PAGE. **a**. The amount of tyrosine phosphorylated HA-SHC was visualized by Western immunoblot analysis with 4G10 anti-phosphotyrosine antibody. **b.** The amount of HA-SHC in each precipitate was visualized by Western immunoblot analysis with anti-SHC polyclonal antibody.

Figure 7. Y845F mutant receptor retains its ability to phosphorylate MAPK. Cos-7 cells were transfected with plasmid DNA encoding flag-MAPK and either Y845F or *wt* EGFR, cultured for two days, serum starved overnight, and left untreated or treated with 100 ng/ml EGF for 10 min. Extracts were immunoprecipitated with anti-flag M2 affinity

gel and resolved by SDS/PAGE. **a.** The amount of tyrosine phosphorylated flag-MAPK was visualized by Western immunoblot analysis with anti-active MAPK antibody. **b.** The amount of flag-MAPK in each precipitate was visualized by Western immunoblot analysis with B3B9 anti-MAPK antibody.

Figure 8. PP1 inhibits soft agar colony formation of several breast tumor cell lines. Values for number of colonies are the mean ± SEM of at least 3 experiments in which 10^5 cells of each cell line were seeded per plate in triplicate and treated with either 10μg/ml PP1 or an equivalent volume of DMSO every 3-4 days.

Figure 9. Kinase-inactive c-Src dose-dependently inhibits soft agar colony formation of MDA-MB468 breast tumor cells. **a.** Western immunoblot analysis of MDA-MB468 and MCF-7 clonal cell lines stably overexpressing kinase deficient (K-) c-Src. Fold overexpression was estimated by densitometry analysis. **b.** Values for number of colonies are the mean ± SEM of at least 3 experiments in which 10^5 cells of each clone were seeded per plate in triplicate.

Figure 10. Myc-tagged c-Src domain constructs. Various c-Src domains were PCR amplified and cloned into a pcDNA3.1 myc epitope vector.

Figure 11. Expression of myc-tagged c-Src domains in MDA-MB468 breast tumor cells. Western immunoblot analysis of MDA-MB468 clonal cell lines stably overexpressing myc-tagged c-Src domains.

Figure 12. The c-Src kinase domain and SH2 domain inhibit soft agar colony formation of MDA-MB468 breast tumor cells. Values for number of colonies are the mean ± SEM of at least 3 experiments in which 10^5 cells of each clone were seeded per

plate in triplicate. The cells expressing the unique, SH3, SH2 and N-term domains are expressed as the mean of two clones.

Figure 13. The c-Src kinase domain and SH2 domain inhibit BrdU incorporation in MDA-MB468 breast tumor cells. MDA-MB468 breast tumor cells were transfected with plasmid DNA encoding myc-tagged c-Src domains, cultured for one day, and incubated with 100 μM BrdU for 17hrs. Cells were fixed and co-stained for myc-tag expression and BrdU incorporation. Results are expressed as the mean percent ± SEM of cells expressing EGFR that were positive for BrdU incorporation. Approximately 100 cells were analyzed for each variable in 3 independent experiments.

Table I. Kinase-deficient c-Src completely ablates tumor formation in nude mice in 10T1/2 clones.

Cell Line	**Tumor Volume (mm^3)**
EGFR	295 ± 124*
$EGFR/K^+_9$	1592 ± 598*
$EGFR/K^-$	0 *†

* Mean tumor volume $\pm$ SEM of 8 individual sites was measured at day 52 after subcutaneous injection with 10^7 cells.

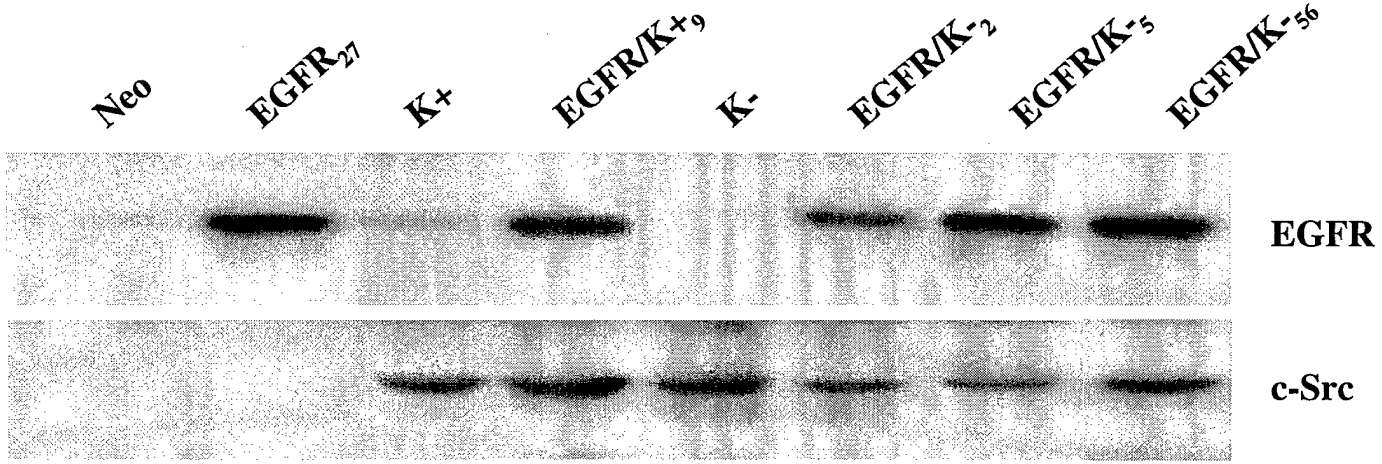
† EGFR/K- represents the mean tumor volume of two individual clones, $EGFR/K^-_{41}$ and $EGFR/K^-_{56}$.

Table II. Kinase deficeint c-Src dose-dependently inhibits tumor formation of MDA-MB-468 breast tumor cell line.

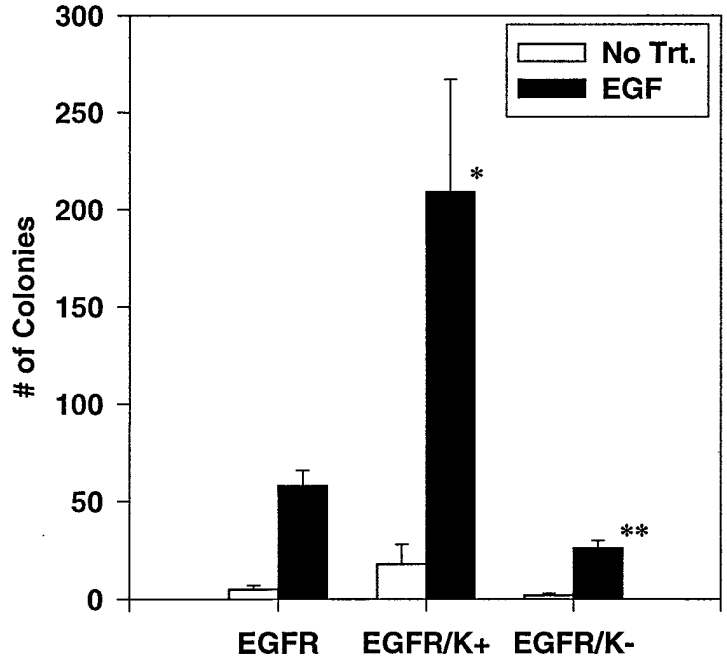
Cell Line	Tumor Size*
MDA-MB-468	228 ± 45
468 K^-_2	102 ± 21
468 K^-_8	56 ± 8

* Mean tumor volume ± SEM of 8 individual sites was measured at day 23 after subcutaneous injection with 10^7 cells.

a.


Neo
EGFR27
K+
EGFR/K+9
K-
EGFR/K-2
EGFR/K-5
EGFR/K-56
EGFR
c-Src

b.


300
250
200
150
100
50
0
# of Colonies
No Trt.
EGF
*
**
EGFR
EGFR/K+
EGFR/K-

c.

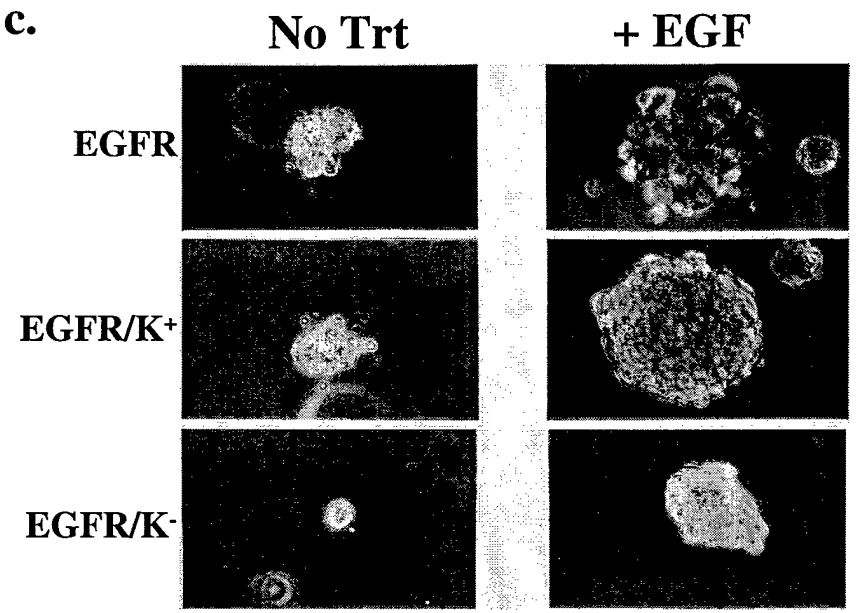

No Trt
+ EGF
EGFR
EGFR/K+
EGFR/K-

Figure 1

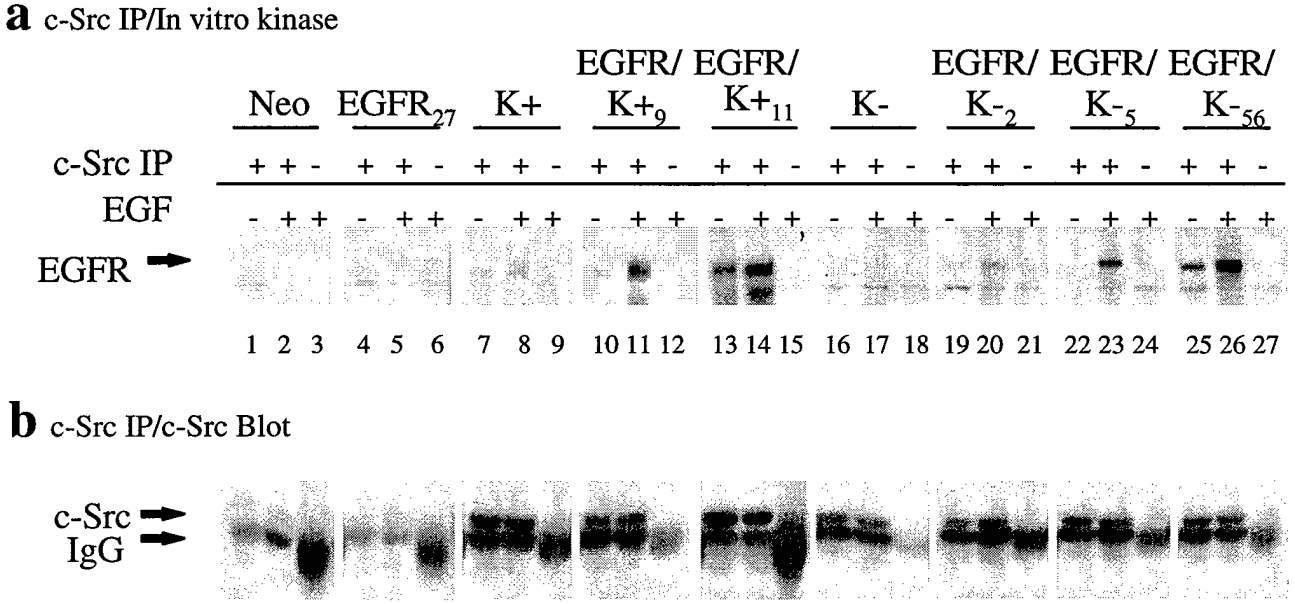

a c-Src IP/In vitro kinase
Neo
EGFR27
K+
EGFR/ K+9
EGFR/ K+11
K-
EGFR/ K-2
EGFR/ K-5
EGFR/ K-56
c-Src IP
EGF
EGFR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
b c-Src IP/c-Src Blot
c-Src
IgG

Figure 2

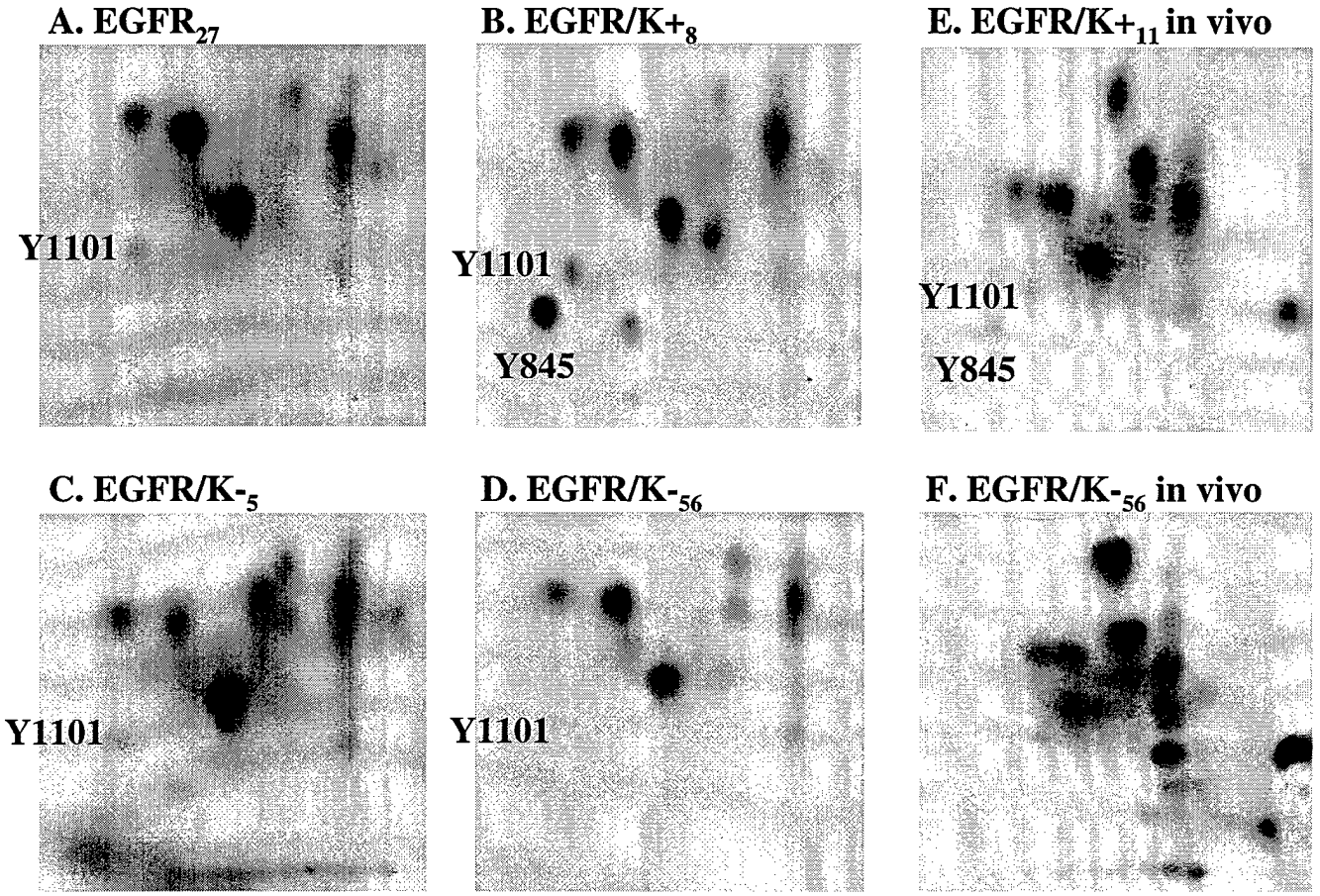

A. EGFR27
Y1101
B. EGFR/K+8
Y1101
Y845
E. EGFR/K+11 in vivo
Y1101
Y845
C. EGFR/K-5
Y1101
D. EGFR/K-56
Y1101
F. EGFR/K-56 in vivo

Figure 3

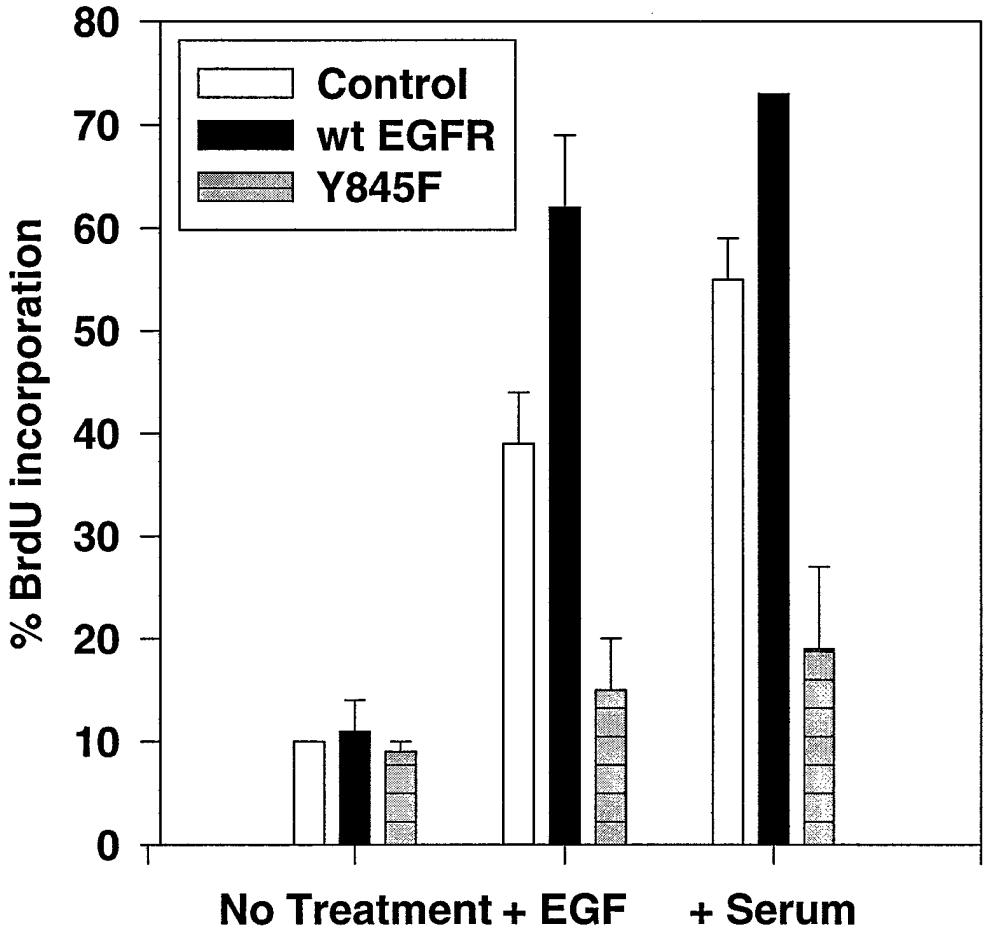
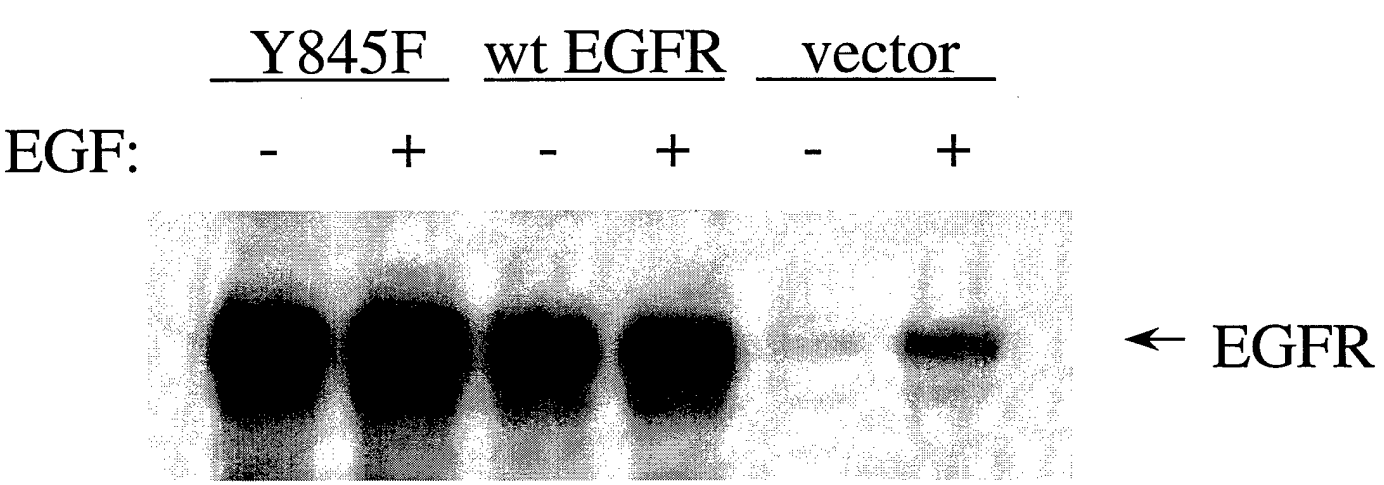

80
70
60
50
40
30
20
10
0
% BrdU incorporation
Control
wt EGFR
Y845F
No Treatment
+ EGF
+ Serum

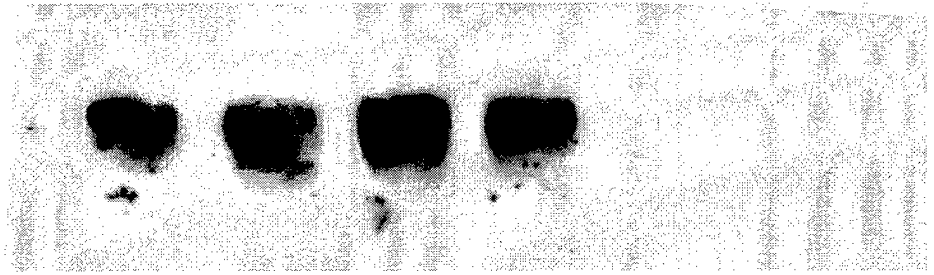
Figure 4

a.



EGFR IP/kinase assay

b.



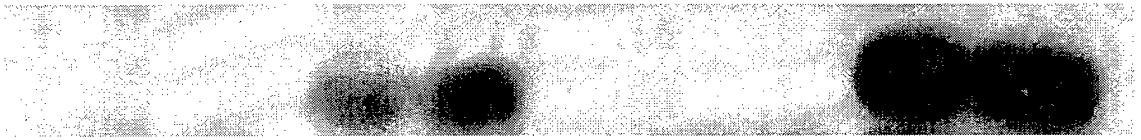
EGFR IP/EGFR blot

Figure 5

a.

EGFR:	-	-	Wt	Y-F	-	-	Wt	Y-F
HA-SHC:	-	+	+	+	-	+	+	+

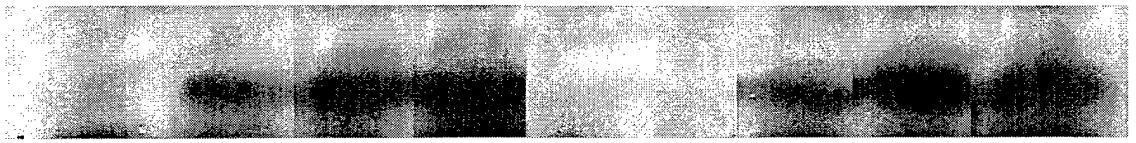
EGF:	-	-	-	-	+	+	+	+



← SHC

HA IP / P-Tyr blot

b.



← SHC

HA IP / SHC blot

Figure 6

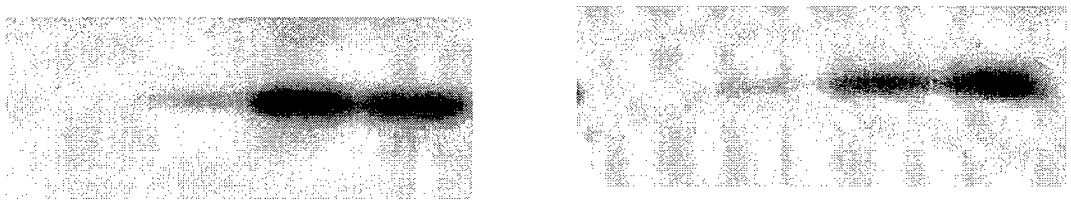
a.

EGFR:	-	-	Wt	Y-F		-	-	Wt	Y-F
Flag - ERK2:	-	+	+	+		-	+	+	+

EGF:	-	-	-	-		+	+	+	+

Flag IP / Phospho-MAPK blot

b.



Flag IP / MAPK blot

Figure 7

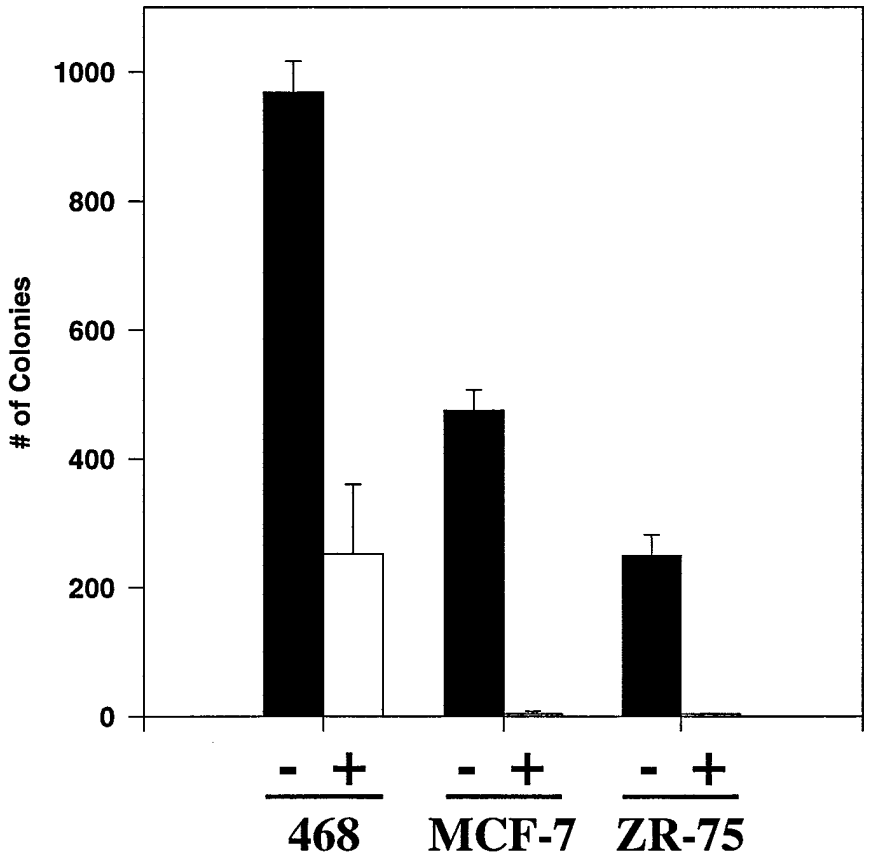
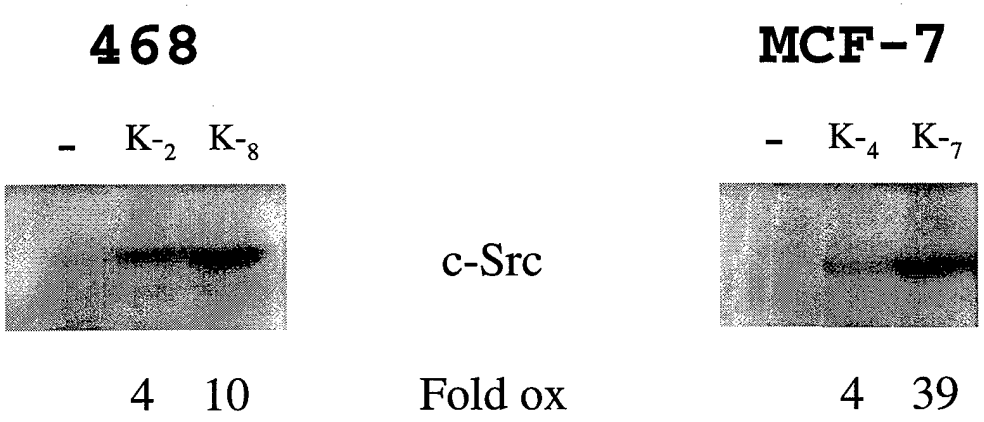


Figure 8

a.


468
MCF-7
- K-2 K-8
- K-4 K-7
c-Src
4 10
Fold ox
4 39

b.

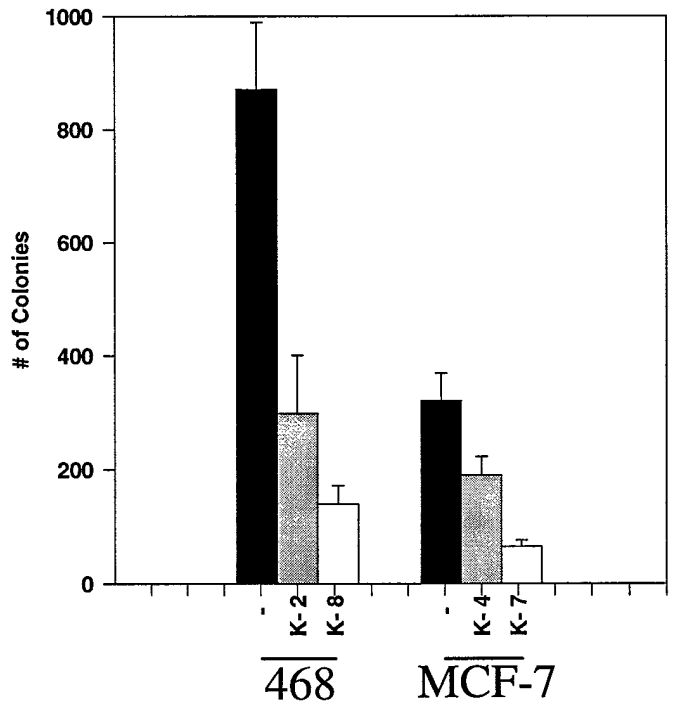

1000
800
600
400
200
0
# of Colonies
-
K-2
K-8
-
K-4
K-7
468
MCF-7

Figure 9

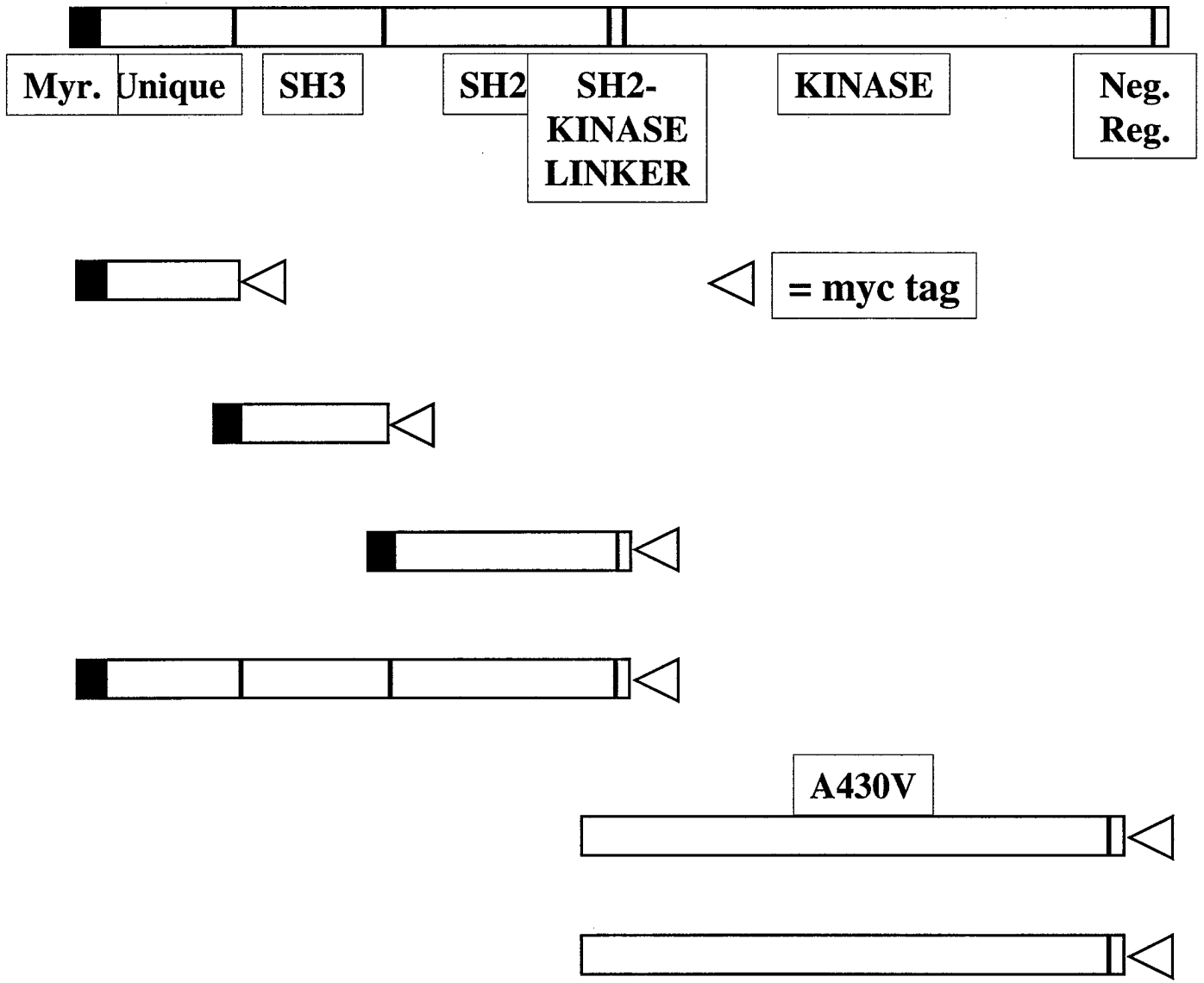

Myr.
Unique
SH3
SH2
SH2-
KINASE
LINKER
KINASE
Neg.
Reg.
= myc tag
A430V

Figure 10

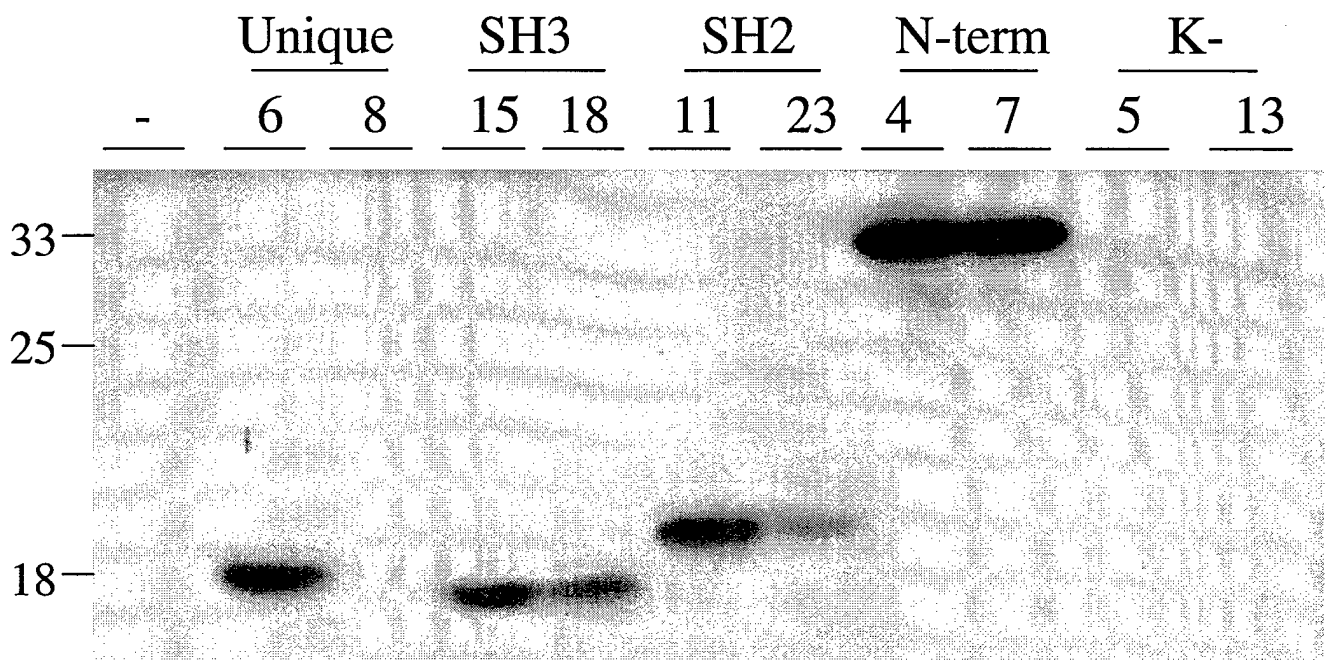


Figure 11

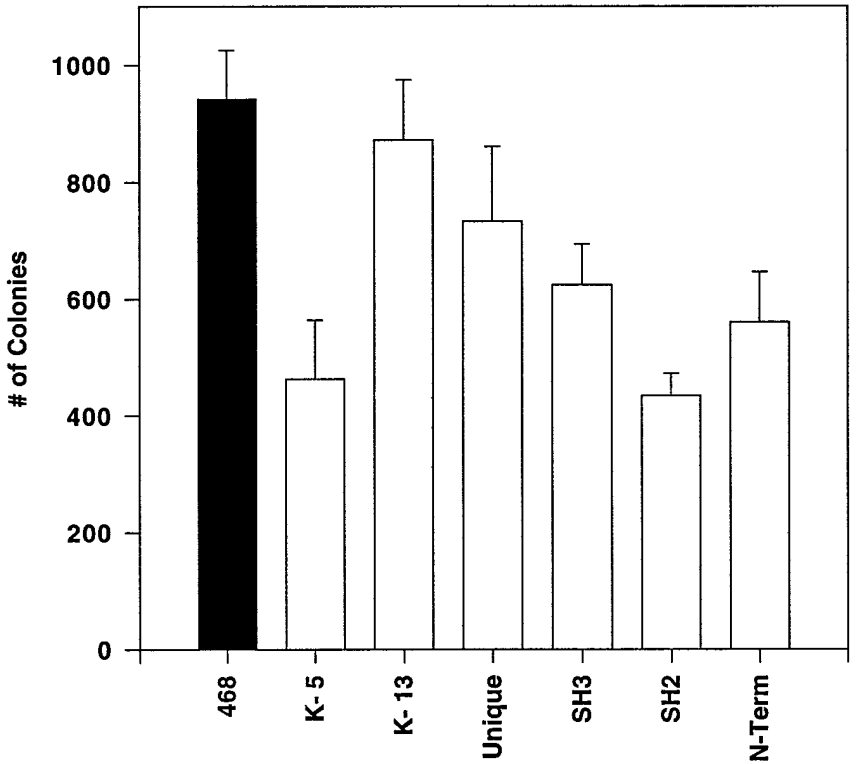

# of Colonies
1000
800
600
400
200
0
468
K-5
K-13
Unique
SH3
SH2
N-Term

Figure 12

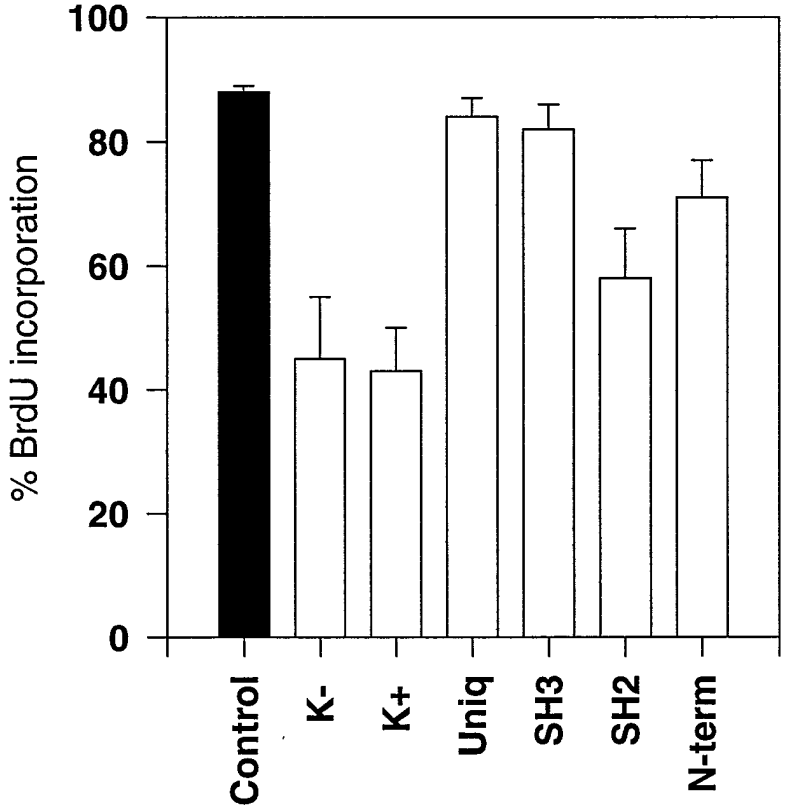

% BrdU incorporation
100
80
60
40
20
0
Control
K-
K+
Uniq
SH3
SH2
N-term

Figure 13